Steroids 77 (2012) 91–99

Contents lists available at SciVerse ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Brassinosteroids and analogs as neuroprotectors: Synthesis and structure–activity relationships

Jihane Ismaili, Martin Boisvert, Fanny Longpré, Julie Carange, Céline Le Gall, Maria-Grazia Martinoli, Benoit Daoust *

Département de Chimie-Biologie, Groupe de Recherche en Neuroscience, Université du Québec à Trois-Rivières, 3351 Boulevard des Forges, C.P. 500, Trois-Rivières, Québec, Canada G9A 5H7

ARTICLE INFO

Article history:
Received 4 August 2011
Received in revised form 18 October 2011
Accepted 20 October 2011
Available online 28 October 2011

Keywords:
Brassinosteroids
Structure–activity relationship
Neuroprotection
MPP^+
Dopaminergic cells
Parkinson's disease

ABSTRACT

We have demonstrated previously that the brassinosteroid (BR) 24-epibrassinolide exerts neuroprotective effects deriving from its antioxidative properties. In this study, we synthesized 2 natural BRs and 5 synthetic analogs and analyzed their neuroprotective actions in neuronal PC12 cells, against 1-methyl-4-phenylpyridinium (MPP^+), a neurotoxin known to induce oxidative stress and degenerescence of dopaminergic neurons characteristic of Parkinsonian brains. We also tested the neuroprotective potential of 2 commercially available BRs. Our results disclosed that 6 of the 9 BRs and analogs tested protected neuronal PC12 cells against MPP^+ toxicity. In addition, our structure–activity study suggests that the steroid B-ring and lateral chain play an important role for their neuroprotective action.

1. Introduction

BRs are highly oxygenated steroids isolated from several vegetables, including *Vicia faba* seeds and pollen [1–3]. To date, about 60 natural BRs have been identified, such as 24-epibrassinolide (**1**) and homocastasterone (**2a**) (Fig. 1), both oxygenated in positions 2, 3, 6, 22 and 23 [4]. Several BR non-natural analogs, such as 22S,23S-homocastasterone (**2b**) and 22S,23S-homobrassinolide (**3**) (Fig. 1), have been synthesized [5,6]. Owing to their peculiar structural features, their extremely low abundance in natural sources and potent biological activity [7], BRs have been the subject of numerous synthetic efforts [5,6,8–10]. Their 4 contiguous chiral centers (C-20, C-22, C-23 and C-24) represent major challenges in the synthesis of these steroids. The methods developed by McMorris et al. [5], Mori et al. [6] and Brosa et al. [8] are especially efficient and versatile, allowing the swift preparation of a variety of 29-carbon BRs. All these protocols use stigmasterol as the starting product, and their synthetic scheme can be summarized in 2 main reaction sequences: (i) transformation of the homoallylic alcohol functionality of stigmasterol into 2,3,6- and 3,6-oxygenated moieties, and (ii) standard osmylation of the 22,23-alkene, resulting in 22,23-dihydroxylated steroidal side-chains.

BRs are being studied intensively to understand their role in plant metabolism. Their main physiological effects in plants include regulation of hormonal balance, activation of protein and nucleic acid synthesis, enzyme activity, growth promotion, and, most interestingly, increased resistance to unfavorable environmental factors, stress and diseases (for review see [7]). BRs have also been reported to exert anti-oxidative actions [11–17]. Exogenous application of natural BRs, such as 24-epibrassinolide (**1**), to plants enhances activities of the enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT) and peroxidase, reducing lipid peroxidation [11–15]. Oral administration of homobrassinolide evokes anti-oxidative outcomes in mammals [16]. Recently, we determined that 24-epibrassinolide (**1**) modulates SOD, CAT and glutathione peroxidase (GPx) in mammalian cells [17].

Several neurodegenerative diseases, e.g. Parkinson's disease (PD), are associated with oxidative stress [18]. PD is characterized by the selective degeneration of nigrostriatal dopaminergic neurons, resulting in dopamine (DA) depletion [19]. Numerous studies have demonstrated that, in *post mortem* samples of *substantia nigra pars compacta*, DAergic neurons exhibit markers of oxidative stress, such as lipid peroxidation, DNA oxidative damage, and carbonyl modifications of soluble proteins [20,21]. L-3,4-Dihydroxyphenylalanine (L-dopa), the amino acid precursor of DA, is nowadays the most effective symptomatic treatment of PD [22]. Clinical reports indicate that consumption of *V. faba* beans and seedlings, which contain L-dopa [23,24], has beneficial effects on PD patients

* Corresponding author. Tel.: +1 819 376 5011x3349; fax: +1 819 376 5084.
E-mail address: benoit.daoust@uqtr.ca (B. Daoust).

0039-128X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2011.10.009

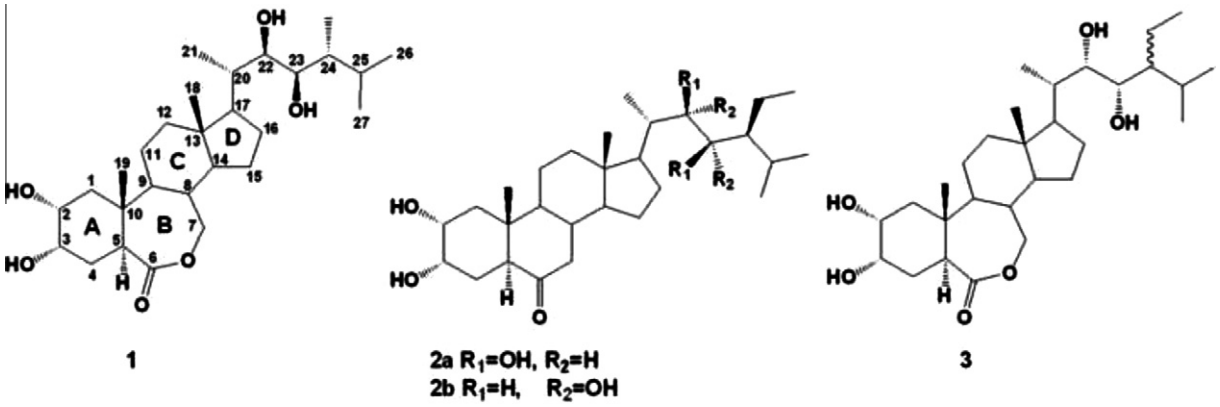


Fig. 1. Structure of 24-epibrassinolide (**1**), homocastasterone (**2**), and 22S,23S-homobrassinolide (**3**).

[25,26]. However, the concentration of L-dopa in *V. faba* is not sufficient to explain the magnitude of the responses observed in PD patients [26]. Thus, it raises the possibility that other compounds in *V. faba* may complement the effect of L-dopa. It is known that *V. faba* contains BRs such as 24-epibrassinolide (**1**), castasterone and brassinolide [1–3]. Recently, we established that 24-epibrassinolide (**1**) is neuroprotective of nerve growth factor (NGF)-differentiated PC12 (neuronal) cells against MPP^+-induced toxicity [17]. MPP^+ is the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a toxin serving extensively to reproduce PD in animal models [27]. This neurotoxin is known to act as an in vitro and in vivo oxidant [28]. MPP^+ is selectively taken up by DAergic neurons via high-affinity DA transporters expressed in NGF-differentiated PC12 cells [29,30]. The neurotoxin is actively transported into mitochondria where it interferes with mitochondrial respiration through complex I inhibition [31–33]. It evokes elevated levels of reactive oxygen species (ROS) in MPP^+-treated neuronal cells [34–36] and neuronal cell death by apoptosis [37,38]. We recently reported that 24-epibrassinolide (**1**) modulates SOD, CAT and GPx activities and reduces MPP^+-induced apoptosis and intracellular ROS in neuronal PC12 cells [17].

The aim of the present investigation study was to evaluate the neuroprotective effects of natural BRs and synthetic analogs and provide new insights into their structure–activity relationships as neuroprotective molecules in a well-known in vitro model of PD, NGF-differentiated PC12 cells [29,39]. We followed the protocol of Brosa et al. to prepare BRs for this study [8]. This strategy was particularly well-suited for our work since it allowed us to freely functionalize steroid A and B rings as well as C20–C29 side-chain. We were, therefore, able to synthesize 7 BRs with different levels of oxygenation. We demonstrated that some of these molecules were neuroprotective against MPP^+-evoked toxicity. Structure–activity analysis revealed the importance of lateral chain and B ring functionalization for neuroprotection. We also noted that BR hydroxyl group configurations were not crucial for neuroprotection. Overall, our findings clearly indicate that BRs and analogs are new protective molecules against MPP^+-induced toxicity. Therefore, they might be regarded as novel candidates to investigate the outcomes of complementary and/or preventive therapies in neurodegenerative diseases.

2. Experimental

2.1. General

All reagents, including 24-epibrassinolide (**1**) and 22S,23S-homobrassinolide (**3**), were purchased from Sigma–Aldrich (Oakville, ON, Canada) unless noted otherwise. All solvents (Fisher Scientific, Ottawa, ON, Canada) were ACS-certified, distilled and dried prior to use. Reactions requiring anhydrous conditions were conducted under positive nitrogen atmosphere in oven-dried glassware, and reaction flasks were fitted with rubber septa for the introduction of substrates and reagents via standard syringe techniques. Flash chromatography was performed on Merck silica gel 60 on Siliaflash P60 (0.040–0.063 mm, Silicycle, Quebec, QC, Canada) under compressed air pressure. Analytical thin-layer chromatography (TLC) was carried out on pre-coated (0.25 mm) Merck silica gel F54 plates (VWR, Ville Mont-Royal, Qc, Canada) or on silica gel 60 (0.25 mm, Silicycle, Quebec, QC, Canada) and developed with an acid solution of ammonium phosphomolybdate. ^{1}H NMR spectra were recorded on a Varian 200 MHz NMR spectrometer with $CDCl_3$ (δ = 7.26 ppm) as reference. ^{13}C NMR spectra were traced at 50.3 MHz with $CDCl_3$ (δ = 77.1 ppm) as reference. For acetate **9**, NMR spectra were charted on a Varian 600-MHz spectrometer. The data reflect the following: chemical shift in ppm, multiplicity (d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, dd = doublet of doublets, dt = doublet of triplets, td = triplet of doublets), number of protons, coupling constants in Hertz and assignation (if possible). IR spectra were recorded by Nicolet Impact 420 spectrophotometer. Low resolution mass spectra (LRMS) were recorded on an Agilent Technologies GC system 6890 N and mass detector 5973 with helium as carrier gas. High resolution mass spectra (HRMS) were measured in the electrospray (ESI) mode on an HPLC 1200 system with a TOF 6210 detector from Agilent Technologies. Melting points (mp) were recorded on an Electrothermal apparatus and were uncorrected. If compounds were recrystallized prior to determination of their melting points, the re-crystallization solvent is indicated in brackets.

2.2. (22E,24S)-3α,5-cyclo-stigmast-22-en-6-one (*5*)

Compound **5** was prepared in 3 steps from stigmasterol (**4**) according to Brosa's method [8]. Under N_2 atmosphere and anhydrous conditions, 5.25 g (12.1 mmol) of stigmasterol (**4**) 95% (Acros Organics, Fisher Scientific, Ottawa, ON, Canada) were dissolved in 60 mL of toluene. To this solution, 13.0 mL (93.1 mmol, 7.7 eq) of triethylamine were added with a syringe. The mixture was then cooled to 0 °C and 2.8 mL (36.1 mmol, 3.0 eq) of $MeSO_2Cl$ were included drop-wise over 10 min. After stirring at 0 °C for 1.5 h, the resulting yellow solution was diluted with water and extracted with toluene (3X). The organic layers were washed with saturated aq. $NaHCO_3$ (2X) and brine, then dried over $MgSO_4$. Evaporation of the solvent provided 5.84 g (97%) of the mesylate. This step was repeated several times, and the yields obtained were 95–97%. ^{1}H- and ^{13}C NMR data on the crude compound were consistent with data in the literature [8].

4.30 g (8.78 mmol) of mesylate were suspended in 80 mL of water and 240 mL of acetone. 1.09 g (13.2 mmol, 1.2 eq) of $KHCO_3$ was then added and the suspension was heated under reflux for 6 h to achieve i-sterol rearrangement. The solution was allowed to reach room temperature and extracted with EtOAc (3X). The organic layers were washed with water, saturated aq. $NaHCO_3$ and brine, then dried over $MgSO_4$. Evaporation of the solvent provided 3.34 g (93%) of yellow oil. This step was repeated several times and the yields obtained were 87–93%. Note that the ^{1}H- and ^{13}C NMR data on the crude compound were consistent with data in the literature [8].

3.16 g (7.65 mmol) of crude alcohol were dissolved in 80 mL of acetone. The solution was stirred and cooled to 0 °C in an ice bath. Jones reagent (8 N–CrO_3: 5.33 g of CrO_3 were dissolved in 4.4 mL of concentrate H_2SO_4, then water was added to obtain a 20 mL solution) was included dropwise until the mixture became reddish (∼3 mL). The reaction was stirred at 0 °C for 5 min. and the solvent was removed under reduced pressure. The green residue was diluted with water and extracted with EtOAc (3X). The organic layers were combined, washed with saturated aq. $NaHCO_3$ (2X) and brine, then dried over $MgSO_4$. Evaporation of the solvent provided 2.82 g (83%) of pure **5**. This step was repeated several times, and the yields obtained were 78–83%. mp lit. 98–99 °C [8] exp. (EtOH) 95–97 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 5.16 (dd, 1H, J = 8 and 15 Hz, H-22), 5.08 (dd, 1H, J = 8 and 15 Hz, H-23), 2.42 (1H, dd, J = 8 and 14 Hz, H-7α), 2.10–1.10 (m, 25H), 1.03 (s, 3H, H-19), 1.02 (d, 3H, J = 6 Hz, H-21), 0.98–0.78 (m, 9H), 0.73 (s, 3H, H-18). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm) 210.1 (C-6), 138.3 (C-22), 129.7 (C-23), 57.7, 56.1, 51.5, 47.0, 46.5, 46.3, 45.0, 42.8, 40.7, 39.8, 35.6, 35.0, 33.7, 32.1, 29.1, 26.1, 25.6, 24.4, 23.1, 21.4, 21.3, 19.9, 19.2, 12.5, 12.4, 11.9. IR (KBr, ν (cm^{-1})) 2943, 2856, 1685, 1478, 1369, 1298, 1167, 966, 922. LRMS (*m/z*, relative intensity) 410 (M^+) (100), 392 (10), 367 (70), 298 (70), 271 (75). Note that ^{1}H- and ^{13}C NMR data on this compound were consistent with data in the literature [8].

2.3. (22E,24S)-stigmasta-2,22-dien-6-one (**6**)

Under inert atmosphere, a mixture of pentacyclic ketone **5** (4.88 g, 11.87 mmol), pyridinium chloride (0.51 g, 5.94 mmol, 0.50 eq), dry LiBr (0.69 g, 5.94 mmol, 0.50 eq) and dimethylacetamide (DMA, 50 mL) was heated under reflux conditions for 4 h. The reaction was then cooled to 0 °C to initiate crystallization. The pure product was collected by filtration. The filtrate was also cooled to 0 °C to induce further crystallization, and the product was again filtered. This step was repeated 2 more times until no crystallization were apparent. 4.67 g (96%) of pure **6** was obtained. mp lit. 110–111 °C [8] exp. (EtOH) 111–112 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 5.66–5.58 (m, 2H, H-2 and H-3), 5.13 (dd, 1H, J = 8 and 15 Hz, H-22), 5.07 (dd, 1H, J = 8 and 15 Hz, H-23), 2.40–1.20 (m, 26 H), 1.04 (d, 1H, J = 7 Hz, H-21), 1.02–0.80 (m, 9H), 0.71 (s, 3H, H-19), 0.69 (s, 3H, H-18). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm) 212.4 (C-6), 138.3 (C-22), 129.8 (C-23), 125.2 and 124.8 (C-2 and C-3), 57.1, 56.1, 54.1, 53.7, 51.5, 47.3, 42.9, 40.7, 40.3, 39.6, 37.9, 32.1, 29.0, 25.6, 24.2, 22.0, 21.4, 21.3, 19.2, 13.7, 12.5, 12.4. IR (KBr, ν (cm^{-1})) 2961, 2863, 1710, 1460, 1389, 965. LRMS (*m/z*, relative intensity) 410 (M^+) (100), 395 (80), 367 (25), 297 (25), 269 (30). Note that the ^{1}H- and ^{13}C NMR data on this purified compound were consistent with data in the literature [5,8].

2.4. General procedure for preparation of compounds **2a** and **2b**

0.62 g (1.52 mmol) of diene **6** was dissolved in 30 mL of t-BuOH/H_2O (1:1). To this solution was added 1.26 g (9.10 mmol, 6.00 eq) of K_2CO_3, 3.00 g (9.10 mmol, 6.00 eq) of $K_3Fe(CN)_6$, 0.14 g (0.30 mmol, 0.20 eq) of dihydroquinidine-4-chlorobenzoate (DHQD), 0.29 g (3.00 mmol, 2.00 eq) of methanesulfonamide and 0.60 mL (0.06 mmol, 0.04 eq) of a 2.5% OsO_4 solution in *t*-butanol. The mixture was stirred at room temperature for 6 days. 1.20 g of $NaHSO_3$ was then added and the reaction was stirred for another 18 h at room temperature. t-BuOH was removed under reduced pressure, and the residue was extracted with EtOAc (6X). Combined organic layers were washed with water, 0.25 M H_2SO_4 (3X) and brine, then dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel CH_3Cl/EtOH (0 → 6%) to provide the 2 desired compounds, **2a** and **2b**.

2.4.1. (22R, 23R)-homocastasterone (**2a**)

81.9 mg (13.4%). TLC: $CHCl_3$/EtOH (9:1) R_f = 0.21. mp lit. 227–230 °C [40] exp. 223–225 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 4.05 (m, 1H, H-3), 3.66–3.82 (m, 2H), 3.59 (d, 1H, J = 9 Hz), 2.69 (dd, 1H, J = 3.5 and 12.5 Hz, H-5), 2.3 (dd, 1H, J = 4.3 and 12.9 Hz), 0.9–2.2 (m), 0.76 (s, 3H, H-19), 0.68 (s, 3H, H-18). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm) 211.9 (C-6), 74.5 and 72.7 (C-22 and C-23), 68.4 and 68.3 (C-2 and C-3), 56.5, 53.6, 52.5, 50.7, 46.7, 46.3, 42.8, 42.6, 40.2, 39.4, 37.7, 36.9, 30.9, 28.8, 27.6, 26.3, 23.8, 21.2, 19.4, 18.8, 13.6, 13.4, 11.9, 11.9. IR (neat, ν (cm^{-1})) 3493, 3447, 2965, 2940, 2862, 1699. HRMS calculated for $C_{29}H_{51}O_5$ (MH^+): 479.3737; found 479.3723. Note that the ^{1}H- and ^{13}C NMR data on this purified compound were consistent with data in the literature [5].

2.4.2. (22S,23S)-homocastasterone (**2b**)

71.2 mg (11.5%). TLC: $CHCl_3$/EtOH (9:1) R_f = 0.29. mp lit. 204–207 °C [5] exp. 206–208 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 4.05 (m, 1H, H-3), 3.72–3.82 (m, 1H), 3.58–3.62 (m, 2H, H-22 and H-23), 2.68 (dd, 1H, J = 3.5 and 12.5 Hz, H-5), 2.3 (dd, 1H, J = 4.3 and 12.9 Hz), 0.9–2.2 (m), 0.76 (s, 3H, H-19), 0.70 (s, 3H, H-18). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm) 211.9 (C-6), 72.2 and 70.6 (C-22 and C-23), 68.4 and 68.3 (C-2 and C-3), 56.3, 53.7, 52.6, 50.7, 49.6, 46.7, 43.5, 42.5, 42.3, 40.2, 39.4, 37.6, 27.8, 26.9, 26.3, 24.2, 21.7, 21.2, 18.6, 17.7, 14.5, 14.1, 13.5, 11.9. IR (neat, ν (cm^{-1})) 3361, 2948, 2869, 1713. HRMS calculated for $C_{29}H_{51}O_5$ (MH^+): 479.3737; found 479.3723. Note that the ^{1}H- and ^{13}C NMR data on this purified compound were consistent with data in the literature [5].

2.5. (22E,24S)-2α,3α-dihydroxy-5α-stigmast-22-en-6-one (**7**)

0.25 g (0.61 mmol) of diene **6** was suspended in 7.5 mL of acetone/water (9:1) containing 0.18 g (1.52 mmol, 2.50 eq) of 4-methylmorpholine *N*-oxide (NMO). After the addition of 0.20 mL of a 2.5% OsO_4 solution in t-butanol (0.02 mmol, 0.03 eq), the suspension was stirred at room temperature for 12 h. Solid sodium bisulfite was then added in excess to destroy the remaining OsO_4 and stirred at room temperature for 5 h. The resulting mixture was diluted with water and extracted with EtOAc (3X). The organic layers were washed successively with 5% $NaHSO_3$ (2X), saturated aq. $NaHCO_3$ (2X) and brine (2X), then dried over $MgSO_4$. Evaporation of the solvent gave 217 mg of a yellow solid which was first purified by flash chromatography on silica gel ($CHCl_3$/MeOH 2%) and then recrystallized from EtOH to obtain 160 mg of pure **7** (60%) as white filaments. mp lit. 238–240 °C [41] exp. (EtOH) 237–239 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 5.13 (dd, 1H, J = 8 and 15 Hz, H-22), 5.07 (dd, 1H, J = 8 and 15 Hz, H-23), 4.04 (m, 1H, H-3β), 3.75 (m, 1H, H-2β), 2.65 (dd, 1H, J = 3 and 13 Hz, H-5), 2.31 (dd, 1H, J = 4 and 13 Hz), 2.20–1.10 (m, 24H), 1.03 (d, 3H, J = 6 Hz, H-21), 0.74–0.86 (m, 9H), 0.75 (s, 3H, H-19), 0.68 (s, 3H, H-18). ^{13}C NMR (50 MHz, CDCl3) δ (ppm) 212.3 (C-6), 138.2 (C-22), 129.9 (C-23), 68.6 and 68.5 (C-2 and C-3), 57.0, 56.1, 54.0, 51.5, 50.9, 47.0, 43.1, 42.8, 40.6, 40.5, 39.6, 37.9, 32.1, 28.9, 26.5, 25.6, 24.2 21.4, 21.3, 19.2, 13.8, 12.5. IR (KBr, ν (cm^{-1})) 3384, 2954, 2856, 1712, 1451, 1374, 1048, 966. LRMS (*m/z* relative intensity) (TMS derivative) 573 (M^+-CH_3) (30), 498 (15), 471 (100), 382

(30). HRMS calculated for $C_{29}H_{49}O_3$ (MH^+): 445.3682; found 445.3676. Note that the ^{1}H- and ^{13}C NMR data on this purified compound were consistent with data in the literature [41].

2.6. Homobrassinolide (**8**)

A solution of homocastasterone **2a** (0.106 g, 0.22 mmol) in $CHCl_3$ (10 mL) was added drop-wise to a stirred solution of peroxytrifluoroacetic acid (0.22 mmol, 10 eq) [prepared from 30% aq. H_2O_2 (0.26 mL, 0.22 mmol) and $(CF_3CO)_2O$ (1.58 mL, 1.85 mmol, 5 eq) in $CHCl_3$ (5 mL) at 0 °C]. The reaction mixture was stirred at room temperature for 2 h, diluted with $CHCl_3$ (10 mL), and the resulting solution was washed with H_2O, saturated aq. Na_2CO_3 (2X), saturated $NaHSO_3$ (2X) and brine, then dried over $MgSO_4$. Evaporation of the solvent gave a colorless solid which was recrystallized from EtOAc to obtain 22R,23R-homobrassinolide **8** (70 mg, 65%) mp lit. 249–251 °C [40] exp. (EtOH) 245–247 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 4.05 (m, 3H), 3.71 (m, 2H), 3.57 (d, 1H, J = 8.6 Hz), 3.12 (dd, 1H, J = 5.1 and 12.1 Hz), 0.87–2.2 (m), 0.71 (s, 3H, H-18). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm) 176.2 (C-6), 74.4 and 72.8 (C-22 and C-23), 70.4 and 68.1 (C2 and C-3), 68.1 (C-7), 58.2, 52.5, 51.3, 46.3, 42.5, 41.5, 40.9, 39.6, 39.2, 38.3, 36.9, 31.0, 28.8, 27.6, 24.7, 22.2, 21.2, 19.4, 18.8, 15.5, 13.5, 11.9, 11.7. IR (neat, ν (cm^{-1})) 3436, 2965, 2933, 2837, 1695. HRMS calculated for $C_{29}H_{51}O_6$ (MH^+): 495.3686; found 495.3674. Note that the ^{1}H- and ^{13}C NMR data on this purified compound were consistent with data in the literature [5].

2.7. (22E,24S)-3β-acetoxy-5α-stigmastan-22-en-6-one (**9**)

1.80 g (4.38 mmol, 1.00 eq) of pentacyclic ketone **5** were added to 90 mL of glacial acetic acid. After complete dissolution, 8.8 mL of aqueous 1 M H_2SO_4 were added. The heterogeneous solution was refluxed for 2 h. The solution was then cooled to room temperature, diluted with water and extracted with EtOAc (4X). The organic layers were combined, washed with water (6X), saturated $NaHCO_3$ (2X) and brine (2X). After evaporation of the solvent, the yellow crude product was dissolved in 20 mL of pyridine. 1.85 mL of acetic anhydride and 18 mg of 4-dimethylaminopyridine (DMAP) were then added. After stirring at room temperature for 3 h, the orange mixture was diluted with water and extracted with EtOAc (4X). The organic layers were combined and washed with water (3X), saturated $NaHCO_3$ (2X) and brine (2X). The solution was then dried over $MgSO_4$ and concentrated under vacuum to obtain 1.56 g of **9** (74%) as white crystals. mp lit. 146–147 °C [42] exp. 142–144 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm) 5.13 (dd, 1H, J = 8 and 15 Hz, H-22), 5.03 (dd, 1H, J = 8 and 15 Hz, H-23), 4.66 (m, 1H, H-3α), 2.31 (dd, J = 5 and 13 Hz, 1H, H-5), 2.25 (dd, J = 3 and 13 Hz, 1H, H-7), 2.02 (s, 3H, H-31), 2.03–1.10 (m, 27H), 1.01 (d, J = 6 Hz, 3H, H-21), 0.80 (t, J = 7 Hz, 3H, H-29), 0.78 (d, J = 7 Hz, 3H), 0.77 (s, 3H, H-19), 0.68 (s, 3H, H-18). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm) 211.2 (C-6), 170.6 (C-30), 137.9 (C-22), 129.6 (C-23), 72.8 (C-3), 56.8, 56.5, 55.9, 51.2, 46.6, 42.8, 40.9, 40.4, 39.3, 37.9, 36.4, 31.8, 28.7, 26.8, 26.1, 25.4, 24.0, 21.4, 21.3, 21.1(2), 19.0, 13.0, 12.2(2). ^{1}H-NOESY (a correlation was observed between H-5α (2.31 ppm) and H-3α (4.66 ppm)). IR (KBr, ν (cm^{-1})) 2941, 2876, 1741, 1708, 1478, 1361, 1244, 1023, 960. LRMS (*m/z*, relative intensity) 470 (M^+) (70), 410 (60), 367 (80), 329 (100), 271 (95). HRMS calculated for $C_{31}H_{50}O_3Na$ $(M + Na)^+$: 493.3653; found 493.3652.

2.8. (22E,24S)-3β-hydroxy-5α-stigmastan-22-en-6-one (**10**)

525 mg (1.12 mmol) of acetate **9** were suspended in 45 mL of MeOH. To this suspension were added 2.25 g (5% w/v) of KOH, and the mixture was stirred at room temperature for 2 h. The resulting clear solution was concentrated under vacuum. The yellowish crude product was diluted with water and extracted with EtOAc (3X). The organic layers were combined and washed with water (3X), saturated aqueous NH_4Cl (2X) and brine (2X). The organic layer was dried over $MgSO_4$ and concentrated under vacuum to afford 447 mg of a yellowish solid. This crude product was filtered over a silica gel pad to eliminate colored impurities with $CHCl_3$/EtOH (96:4) as eluent. 425 mg of **10** (89%) were isolated as white crystals. mp lit. 146–147 °C [42] exp. (EtOH) 153–155 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 5.14 (dd, 1H, J = 8 and 15 Hz, H-22), 5.04 (dd, 1H, J = 8 and 15 Hz, H-23), 3.58 (m, 1H, H-3α), 2.30 (dd, J = 5 and 13 Hz, 1H, H-5), 2.23 (dd, J = 3 and 13 Hz, 1H, H-7), 2.10–1.10 (m, 28H), 1.03 (d, J = 6 Hz, 3H, H-21), 0.88–0.74 (m, 9H), 0.66 (s, 3H, H-18). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm) 211.2 (C-6), 138.2 (C-22), 129.8 (C-23), 70.9 (C-3), 57.1, 57.0, 56.1, 54.2, 51.5, 47.0, 43.1, 41.2, 40.6, 39.6, 38.1, 36.9, 32.1, 30.9, 30.3, 29.0, 25.6, 24.3, 21.7, 21.4, 21.3, 19.2, 13.4, 12.5, 12.4. IR (KBr, ν (cm^{-1})) 3411, 2946, 2867, 1708, 1459, 1383, 1062, 968. LRMS (*m/z*, relative intensity) 428 (M^+) (80), 410 (25), 385 (30), 367 (60), 316 (85), 287 (100). HRMS calculated for $C_{29}H_{49}O_2$ (MH^+): 429.3737; found 429.3727.

2.9. (22E,24S)-3α-hydroxy-5α-stigmastan-22-en-6-one (**11**)

Under inert atmosphere, in a 50-mL round-bottomed flask, 400 mg (0.93 mmol) of alcohol **10** was mixed with 978 mg (3.73 mmol, 4.00 eq) of Ph_3P and 0.623 g (3.73 mmol, 4.00 eq) of *p*-nitrobenzoic acid. To this solid mixture were added 20 mL of anhydrous THF with a syringe. The solution was cooled to 0 °C, and 0.75 mL (3.73 mmol, 4.00 eq) of diethyl azodicarboxylate (DEAD) was added drop-wise over 15 min. The orange reaction mixture was stirred at 0 °C for 20 min and at room temperature for 24 h. The solution was then diluted with water and extracted with $CHCl_3$ (3X). The organic layers were combined and washed with water (3X), saturated aqueous $NaHCO_3$ (2X) and brine (2X). The organic layer was dried over $MgSO_4$, filtered and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel with $CHCl_3$ as eluent. 328 mg of nitrobenzoate (61% crude) were obtained as a yellowish solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 8.30 (d, 2H, J = 9 Hz), 8.21 (d, 2H, J = 9 Hz), 5.42 (m, 1H, H-3β), 2.64 (dd, 1H, J = 4 and 12 Hz, H-5), 2.32 (dd, 1H, J = 3 and 13 Hz), 2.10–1.10 (m, 28H), 1.02 (d, 3H, J = 6 Hz, H-21), 0.83–0.75 (m, 9H), 0,70 (s, 3H, H-18). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm) 211.6 (C-6), 164.0, 150.7, 138.2 (C-22), 136.4, 130.8(2), 129.9 (C-23), 123.8(2), 71.2 (C-3), 57.1, 56.2, 54.3, 53.1, 47.0, 43.1, 41.6, 40.7, 39.6, 38.2, 32.9, 32.1, 29.0, 25.6, 25.3, 24.2, 21.4, 21.3, 19.2, 12.7, 12.5.

320 mg (0.55 mmol) of crude nitrobenzoate was suspended in 30 mL of MeOH. 0.375 g (1.25% w/v) of NaOH was added to this suspension. The mixture was stirred at room temperature for 14 h. The yellow solution was concentrated under vacuum, diluted with water and extracted with EtOAc (3X). The combined organic layers were washed with water (3X), saturated aqueous NH_4Cl (2X) and brine (2X). The organic layer was then dried over $MgSO_4$, filtered and concentrated under vacuum. The yellow solid obtained (227 mg) was purified by flash chromatography with $CHCl_3$/MeOH 1% as eluent. 196 mg of **11** (49% over 2 steps) were isolated as white powder. mp exp. (EtOH) 194–196 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 5.14 (dd, 1H, J = 8 and 15 Hz, H-22), 5.04 (dd, 1H, J = 8 and 15 Hz, H-23), 4.17 (m, 1H, H-3β), 2.68 (m, 1H, H-5), 2.25 (dd, J = 3 and 13 Hz, 1H), 2.08–1.08 (m, 28 H), 1.03 (d, J = 6 Hz, 3H, H-21), 0.98–0.72 (m, 9H), 0.68 (s, 3H, H-18). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm) 213.0 (C-6), 138.3 (C-22), 129.8 (C-23), 65.7 (C-3), 57.2, 56.1, 54.1, 51.9, 51.5, 47.1, 43.1, 41.8, 40.6, 39.6, 38.2, 32.1, 31.9, 29.0, 28.4, 27.9, 25.6, 24.2, 21.4, 21.3, 19.2, 12.5, 12.4. IR (KBr, ν (cm^{-1})) 3290, 2941, 2869, 1707, 1465, 1000, 966.

LRMS (*m/z*, relative intensity) 428 (M^+) (20), 410 (80), 367 (60), 316 (55), 271 (100). HRMS calculated for $C_{29}H_{49}O_2$ (MH^+): 429.3733; found 429.3727.

2.10. (24S)-3β-hydroxy-5α-stigmastan-6-one (**12**)

100 mg (0.23 mmol) of sterol **10** were dissolved in 2.5 mL of EtOAc. 20 mg of Pd/C 10% were added to this solution. The reaction flask was equipped with a rubber septum, and hydrogen gas was bubbled directly into the solution for 5 min. The solution was stirred for 3 h under hydrogen atmosphere at room temperature. The preceding step (5 min of bubbling, followed by 3 h of stirring) was repeated twice and stirred for another 14 h under hydrogen atmosphere. The reaction mixture was filtered through Celite. The filtrate was washed twice with water and twice with brine. The organic layer was dried over $MgSO_4$, and concentrated under vacuum. 95 mg of an amorphous solid were obtained. This solid was purified by flash chromatography with $CHCl_3$/MeOH gradient (0 → 6%) as eluent to provide 65 mg of **12** (65%) as a white solid. mp 118–120 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 3.57 (m, 1H, H-3α), 2.38 (dd, 1H, J = 5 and 13 Hz, H-5), 2.20–0.82 (m, 45H), 0.68 (s, 3H, H-18). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm) 211.2 (C-6), 70.9 (C-3), 57.0(2), 56.2, 54.2, 47.0, 46.0, 43.2, 41.2, 39.7, 38.1, 36.9, 36.3, 34.1, 30.9, 30.3, 29.4, 28.3, 26.3, 24.2, 23.3, 21.7, 20.0, 19.2, 18.9, 13.4, 12.2(2). IR (KBr, ν (cm^{-1})) 2395, 2354, 1716, 1531, 1416, 1052, 927. HRMS calculated for $C_{29}H_{51}O_2$ (MH^+): 431.3889; found 431.3887.

2.11. Cell culture and treatments

PC12 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in a controlled environment at 37 °C and 5% CO_2 atmosphere. They were grown in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS), 10% horse serum and gentamicin (50 μg/mL). The culture medium was changed every 2 days, and the cells were seeded at a cellular density of 25,000 cells/cm^2. Neuronal differentiation was induced for 4 days with 50 ng/mL of NGF in RPMI-1640 medium supplemented with 1% FBS. To examine the effect of commercially available BRs (**1** and **3**), synthesized natural BRs (**2a** and **8**) and analogs (**2b**, **7**, **10**, 11 and **12**) on MPP^+-induced cellular death, neuronal PC12 cells were pre-treated with BRs (10^{-9} M), analogs (10^{-9} M) or vehicle (culture medium) for 3 h and exposed to MPP^+ 5 mM for 24 h while maintaining BR or analog concentration at 10^{-9} M. After dose–response experiments, the final concentration of 10^{-9} M 24-epibrassinolide (**1**) was chosen in our previous study as the lowest dose capable of rescuing cells from MPP^+-induced cellular death [17]. For comparative purposes, we worked with the same concentration for all BRs and analogs used in this study. All experiments were performed in phenol red-free medium and charcoal-stripped serum to remove steroids.

2.12. Cytotoxicity measurements

Cytotoxicity was evaluated by colorimetric assay based on the measurement of lactate dehydrogenase (LDH) activity released from damaged cells into the supernatant [43]. LDH is a stable cytoplasmic enzyme present in all cells. It is rapidly released into cell culture supernatant upon damage of the plasma membrane. The amount of enzyme activity detected in culture supernatant correlates with the portion of lysed cells [44,45]. NGF-differentiated PC12 cells were grown and treated in collagen-coated 96-well plates. Then, 100 μL of LDH substrate mixture was added to 50 μL of cell-free supernatant, as described elsewhere [46]. The plate was incubated, protected from light, for 20 min. Absorbance was measured at wavelength 490 nm on a microplate reader (Thermolab System, Franklin, MA). Total cellular LDH was determined by lysing the cells with 1% Triton X-100 (high control); the assay medium served as a low control and was subtracted from all absorbance measurements:

$$\text{Cytotoxicity}(\%) = \frac{(\text{Experimental value} - \text{Low control}) \times 100}{(\text{High control} - \text{Low control})}.$$

2.13. Statistical analysis

Significant differences between groups were determined by 1-way ANOVA, followed by Tukey's post hoc analysis with the GraphPad Instat program, version 3.06, for Windows© (San Diego, CA, www.graphpad.com). All data, at the 95% confidence interval, are expressed as means ± standard error of the mean (SEM) from 3 independent experiments. Asterisks indicate statistical differences between the treatment and respective control condition ($^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0001$); full circles show statistical differences between the treatment and MPP^+ condition ($^{\bullet\bullet}p < 0.01$ and $^{\bullet\bullet\bullet}p < 0001$).

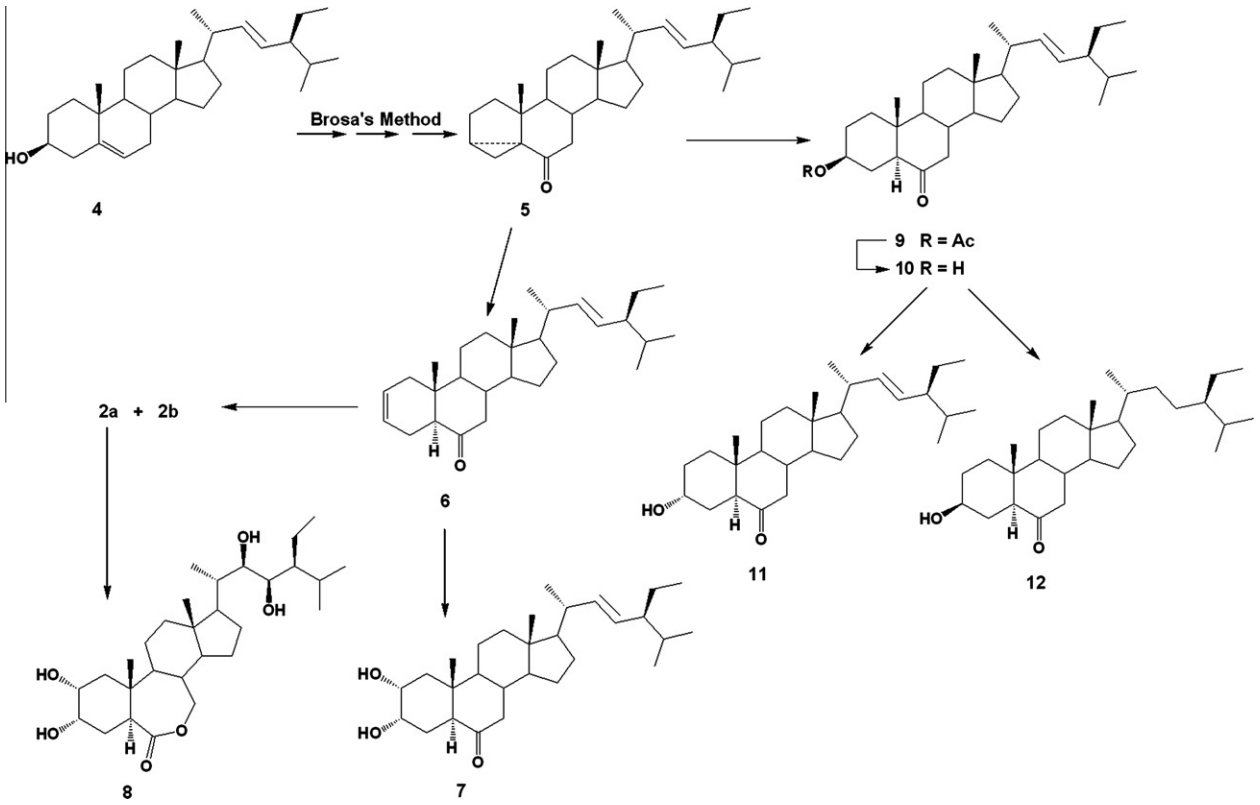
3. Results

3.1. Chemistry

Seven brassinosteroids and analogs were prepared according to the synthetic pathway depicted in Scheme 1. The common starting material for all these compounds was ketone **5** (see Scheme 1), easily prepared from stigmasterol (**4**) with the modified protocol of Brosa et al. [8]. Ketone **5** was then reacted with lithium bromide and pyridinium chloride in refluxing DMA to produce dienone **6** [47]. This reaction consists of acid-catalyzed bromide opening of the 3-membered ring, followed by HBr elimination. We altered the protocol of Brosa et al. slightly to improve the efficiency of their method. Indeed, when preparing dienone **6** from stigmasterol (**4**) (with an overall yield of 56% over 4 steps), Brosa et al. performed 2 laborious time-consuming chromatographic purifications. We eliminated both chromatographic purifications and obtain pure dienone **6** with only simple crystallization in the last step. The overall yield for these 4 steps was also improved to 72%. To form natural 22R, 23R-homocastaterone (**2a**), we treated compound **6** with Sharpless' asymmetric dihydroxylation mixture (OsO_4, $K_3Fe(CN)_6$, K_2CO_3, $MeSO_2NH_2$, DHQD) [5,48]. A 1:1 mixture of natural **2a** and non-natural **2b** was obtained. Separation by flash chromatography yielded pure 22R, 23R-homocastasterone (**2a**) (13% overall yield) and 22S,23S-homocastasterone (**2b**) (11% overall yield). 22R, 23R-homobrassinolide (**8**) was synthesized by Baeyer–Villiger oxidation of **2a** with peroxytrifluoroacetic acid to provide a mixture of 7-oxalactone and 6-oxalactone in a 9:1 ratio from which pure 22R, 23R-homobrassinolide (**8**) was isolated by recrystallization from EtOAc with 65% yield.

We also prepared diol **7** devoid of any OH at positions 22 and 23. Comparing neuroprotective activity of **7** with **2a** and **2b** allowed us to evaluate the importance of oxygenation at positions 22 and 23. Dihydroxylation of dienone **6** with OsO_4/NMO at room temperature for 12 h generated a 10:1 mixture of 2α,3α-diol **7/2β, 3β-diol.** Purification by flash chromatography gave pure compound **7** with 60% yield.

We then moved onto synthesize analogs of the 3-deoxy (homoteasterone) family. Aqueous acetic acid opening of ketone **5** first gave a 2.5:1 mixture of **9** and **10**. Treatment of this crude mixture with DMAP, pyridine and Ac_2O produced **9** with 75% yield. Hydrolysis of **9** under basic conditions yielded alcohol **10** with 89% yield. Note that direct opening of ketone **5** in aqueous basic or acidic conditions (without the presence of any other nucleophiles) did not



Scheme 1. Reagents and conditions: (a) $MeSO_2Cl$, Et_3N, toluene, 0 °C, 1.5 h; $KHCO_3$, acetone/H_2O 3:1, reflux, 6 h; Jones' reagent, acetone, 0 °C, 5 min. (b) PyrHCl, LiBr, DMA, reflux, 4 h. (c) K_2CO_3, $K_3Fe(CN)_6$, OsO_4 cat., DHQD, $CH_3SO_2NH_2$, t-BuOH/H_2O 1:1, rt, 6 days. (d) CF_3CO_3H, $CHCl_3$, rt, 2 h. (e) NMO, OsO_4 cat., acetone/H_2O 9:1, rt, 12 h. (f) H_2SO_4, AcOH, reflux, 2 h; Ac_2O, pyridine, DMAP, rt, 3 h. (g) KOH, MeOH, rt, 2 h. (h) Ph_3P, *p*-nitrobenzoic acid, DEAD, THF, rt, 24 h; NaOH, MeOH, rt, 14 h. (i) H_2, Pd/C 10%, EtOAc, rt, 14 h.

cleanly lead to the desired alcohol **10**. Yields were low and separation was cumbersome. Compound **10** was then taken to prepare 2 other BRs (see Scheme 1). First, the hydroxy group at position 3 was epimerized under standard Mitsunobu conditions [49,50], eliciting 3α steroid **11** with 49% yield. Second, alcohol **10** was cleanly hydrogenated (Pd/C, H_2, EtOAc) to compound **12** (65% yield).

3.2. Cytotoxicity measurements

The ability of BRs and analogs to reverse MPP^+-induced cytotoxicity was investigated by LDH colorimetric assay [35,51]. BRs and analogs, when used alone, did not cause significant cell death of neuronal PC12 cells compared to the control condition (Ctrl., 0.0 ± 0.6%) except for analog **3** **(11.7 ± 4.4%,** Fig. 2B). Treatment with MPP^+ for 24 h induced 28.8 ± 1.6% cell mortality (Fig. 2A). When neuronal PC12 cells received compounds **1**, **2b**, **2a**, **7**, 11 or **10**, cell mortality was significantly reduced (8.5 ± 2.4%; 9.7 ± 2.2%; 13.1 ± 2.3%; 11.9 ± 1.5%; 14.6 ± 2.0 and 11.2 ± 3.6%, respectively) 3 h prior to MPP^+. Analog **3**, BR **8** and analog **12** did not significantly obviate MPP^+-induced cell death (24.2 ± 3.6%; 22.2 ± 3.5% and 24.7 ± 3.1%, respectively).

4. Discussion

Since BRs are present in a large number of plants, animals, including humans, consume BRs in their daily diet. 24-Epibrassinolide (**1**) is found, for example, in *V. faba* [2], and homocastasterone (**2a**) is contained, among others, in Chinese cabbage *Brassica campestris* [52,53], rice *Oryza sativa* [52], rye *Secale cereale* [54], and green tea *Thea sinensis* [52,53]. Our recent study demonstrated the powerful neuroprotective properties of the natural BR 24-epibrassinolide (**1**) against MPP^+-induced oxidative stress and apoptosis [17]. In the present paper, we report that another natural BR, homocastasterone (**2a**), and several analogs (**2b**, **7**, **11**, and **10**) have neuroprotective potential at a nanomolar concentration (10^{-9} M).

The BRs and analogs we investigated, when used alone at nanomolar concentration (10^{-9} M), did not cause significant cell death in neuronal PC12 cells except for analog 22S,23S-homobrassinolide (**3**). BRs, such as 24-epibrassinolide (**1**) and homocastasterone (**2a**), were found, at micromolar concentrations, to inhibit growth and elicit the apoptosis of several human cancer cell lines without affecting the growth of normal cells [55]. Yu et al. observed the micromolar toxicity of 22S,23S-homobrassinolide (**3**), 22R, 23R-homobrassinolide (**8**), 22S,23S-homocastasterone (**2b**) and 22R, 23R-homocastasterone (**2a**) on human cancer cell lines with the (22R,23R) isomer shown to be significantly more toxic than the (22S,23S) isomer [56]. Our study discerned slight toxicity of the (22S,23S) isomer of homobrassinolide (**3**) at nanomolar concentration in neuronal PC12 but not with the (22R,23R) isomer (Fig. 2B).

Strong as our results are with 9 BRs and analogs, we discuss here the influence of functionalization and stereochemistry of these phytosterols on neuroprotection. First, we noted that oxygenation of the B ring greatly influenced BRs neuroprotection against MPP^+ toxicity. Indeed, while ketones **2a** and **2b** possessed neuroprotective activity, lactones **3** and **8** did not present any (Fig. 2A). Compound **1**, differing by only 1 carbon at position 24 (ethyl vs methyl) from **3** and **8**, displayed neuroprotection (Fig. 2A). Therefore, a lactone in the B ring does not completely obviate neuroprotection against MPP^+ toxicity. Second, stereochemistry at positions 22 and 23 did not influence the neuroprotective effect of homocastasterone. Indeed, natural homocasterone **2a** and non-natural analog **2b** showed similar activity.

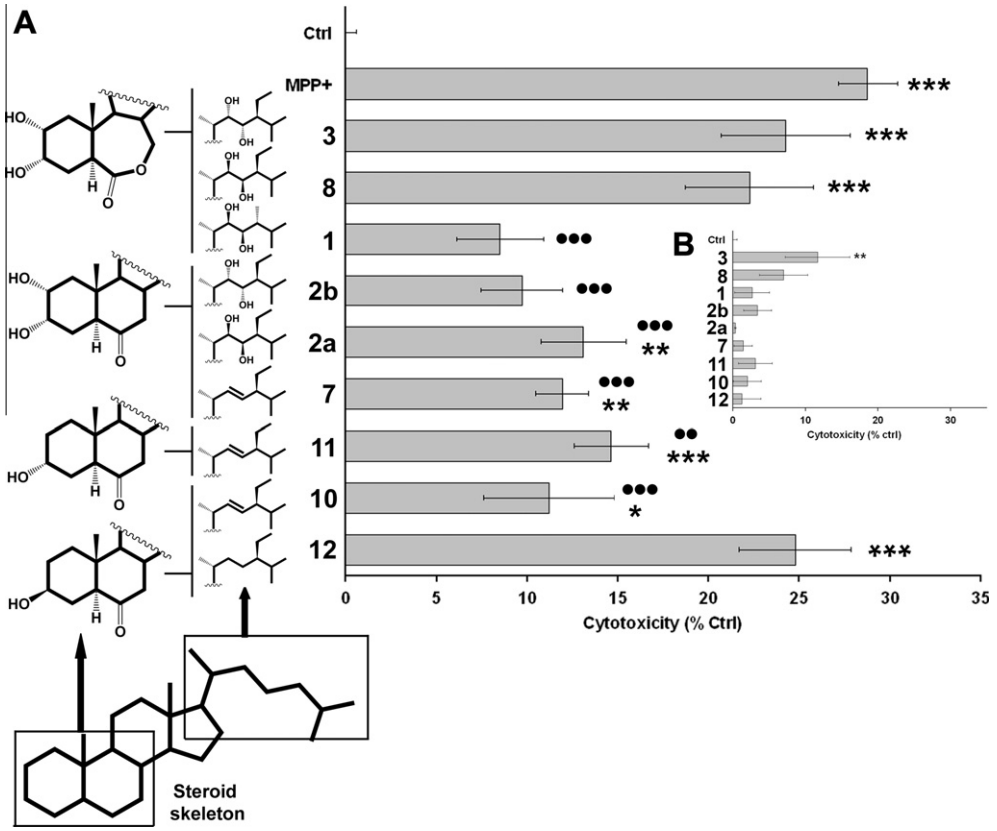


Fig. 2. Cytotoxicity measurement in neuronal PC12 cells by colorimetric assay based on LDH activity. The absorbance value obtained for the untreated control was subtracted from all other values, as described in the Section 2. The data are expressed as percentages of values of untreated control cells and are means ± SEM $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs Ctrl. and $^{\bullet\bullet}p < 0.01$, $^{\bullet\bullet\bullet}p < 0.001$ vs MPP^{+}. (A) Effect of BRs or analogs against MPP^{+}-induced cytotoxicity. Neuronal cells were pre-treated with BRs, analogs (10^{-9} M) or vehicle for 3 h and then exposed to MPP^{+}(5 mM) or not for 24 h. (B) Neurotoxicity of BRs or analogs alone in neuronal PC12 cells. Neuronal cells were treated with BRs or analogs (10^{-9} M) or vehicle for 27 h.

Third, the impact of lateral chain oxygenation on neuroprotection was studied with unsaturated compound **7** in which positions 22 and 23 were not hydroxylated. Neuroprotection similar to hydroxylated BRs **2a** and **2b** was observed (Fig. 2A). Analog **10**, which is unsaturated in 22 and 23, manifested neuroprotection while its saturated counterpart **12** was inactive (Fig. 2A). Neuroprotection seems to be dependent on the presence of hydroxyl groups or unsaturation at positions 22 and 23. Fourth, we analyzed the effect of hydroxylation of the A ring in positions 2 and 3. Comparison of neuroprotection by analogs **7** and **11** demonstrated that the hydroxyl group in position 2 was not important for neuroprotection against MPP^{+}-induced toxicity. Stereochemistry at position 3 did not influence neuroprotection, as observed by comparing neuroprotection of analog **11** (3S isomer) with analog **10** (3R isomer).

BRs should be regarded as interesting molecules for the prevention or treatment of neurodegenerative disease. In this study, several natural BRs and analogs were found to protect neuronal cells against MPP^{+}-induced toxicity. In previous experiments, we associated neuroprotection by 24-epibrassinolide (**1**) against MPP^{+}-induced toxicity with antioxidative and anti-apoptotic properties [17]. Also, recent animal and human studies have shown that phytosterols get through the blood–brain–barrier (BBB) and accumulate in the brain [57–59]. Whether this affects neurocognitive functioning and mental well-being in humans was assessed in hypercholesterolemic individuals, and it was determined that long-term (85 weeks) use of plant sterols or stanols (2.5 g/d) has no impact on neurocognitive functioning or mood [60]. Muthuraman et al. reported that homobrassinolide modulated gene expression in the rat brain, indicating that phytosterol from the BR family or metabolites might pass through the BBB [61]. Finally, BRs have an interesting safety profile. 24-Epibrassinolide (**1**) innocuity was investigated in mice and rats. Acute toxicity (LD50) was more than 1000 mg/kg for mice by oral administration, and more than 2000 mg/kg for rats both orally and dermally [7,62]. The teratogenic potential of homobrassinolide **8** was evaluated in Wistar rats and it was concluded that **8** is non-teratogenic at doses as high as 1000 mg/kg [63]. With our results demonstrating the neuroprotective effect of BRs on neuronal cell cultures and studies showing possible passage of BRs through the BBB with their interesting safety profile, these compounds should thus be regarded as novel molecules in complementary and/or preventive therapies of neurodegenerative diseases and should be assessed in animal models of such disorders.

Acknowledgments

Financial support from Fonds Québécois de la recherche sur la nature et les technologies (FQRNT) is acknowledged. We are thankful to Isabelle Rheault from Université du Québec à Montréal for performing the HRMS.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2011.10.009.

References

[1] Park K-H, Yokota T, Sakurai A, Takahashi N. Occurence of castasterone, brassinolide and methyl 4-chloroindole-3-acetate in immature *Vicia faba* seeds. Agric Biol Chem 1987;51:3081–6.
[2] Ikekawa N, Nishiyama F, Fujimoto Y. Identification of 24-epibrassinolide in bee pollen of the broad bean, *Vicia faba* L. Chem Pharm Bull 1988;36:405–7.
[3] Gamoh K, Omote K, Okamoto N, Takatsuto S. High-performance liquid chromatography of brassinosteroids in plants with derivatization using 9-phenanthreneboronic acid. J Chromatogr 1989;469:424–8.
[4] Bajguz A, Tretyn A. The chemical characteristic and distribution of brassinosteroids in plants. Phytochemistry 2003;62:1027–46.
[5] McMorris TC, Patil PA, Chavez RG, Baker ME, Clouse SD. Synthesis and biological activity of 28-homobrassinolide and analogs. Phytochemistry 1994;36:585–9.
[6] Mori K, Sakakibara M, Ichikawa Y, Ueda H, Okada K, Umemura T, et al. Synthesis of (22S,23S)-homobrassinolide and brassinolide from stigmasterol. Tetrahedron 1982;38:2099–109.
[7] Khripach V, Zhabinskii V, De Groot A. Twenty years of brassinosteroids: steroidal plant hormones warrant better crops for the XXI century. Ann Bot 2000;86:441–7.
[8] Brosa C, Nusimovich S, Peracaula R. Synthesis of new brassinosteroids with potential activity as antiecdysteroids. Steroids 1994;59:463–7.
[9] Khripach VA, Zhabinskii VN, de Groot AE. Brassinosteroids: a new class of plant hormones. San Diego, CA: Academic Press; 1999.
[10] Clutter HG, Yokota T, Adam G. Brassinosteroids: chemistry, bioactivity and applications. Washington, DC: American Chemical Society; 1991.
[11] Mazorra LM, Nùnez M, Hechavarria M, Coll F, Sànchez-Blanco MJ. Influence of brassinosteroids on antioxidant enzymes activity in tomato under different temperatures. Biol Plant 2002;45:593–6.
[12] Ali B, Hayat S, Fariduddin Q, Ahmad A. 24-Epibrassinolide protects against the stress generated by salinity and nickel in *Brassica juncea*. Chemosphere 2008;72:1387–92.
[13] Özdemir F, Bor M, Demiral T, Türkan I. Effects of 24-epibrassinolide on seed germination, seedling growth, lipid peroxidation, proline content and antioxidative system of rice (*Oryza sativa* L.) under salinity stress. Plant Growth Regul 2004;42:203–11.
[14] Hayat S, Ali B, Hasan SA, Ahmad A. Brassinosteroid enhanced the level of antioxidants under cadmium stress in *Brassica juncea*. Environ Exp Bot 2007;60:33–41.
[15] Arora N, Bhardwaj R, Sharma P, Arora HK. 28-homobrassinolide alleviates oxidative stress in salt-treated maize (*Zea mays* L.) plants. Braz J Plant Physiol 2008;20:153–7.
[16] Muthuraman P, Srikumar K. A comparative effect of homobrassinolide and giberellic acid on lipid peroxidation and antioxidant defense in alloxan induced diabetic male albino rats. J Enzyme Inhib Med Chem 2009;24:1122–7.
[17] Carange J, Longpre F, Daoust B, Martinoli MG. 24-Epibrassinolide, a phytosterol from the brassinosteroid family, protects dopaminergic cells against MPP^+ - induced oxidative stress and apoptosis. J Toxicol 2011;2011:392859.
[18] Mariani E, Polidori MC, Cherubini A, Mecocci P. Oxidative stress in brain aging, neurodegenerative and vascular diseases: an overview. J Chromatogr B 2005;827:65–75.
[19] Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. Neuron 2003;39:889–909.
[20] Olanow CW. The pathogenesis of cell death in Parkinson's disease – 2007. Mov Disord 2007;22(Suppl. 17):S335–42.
[21] Zhou C, Huang Y, Przedborski S. Oxidative stress in Parkinson's disease: a mechanism of pathogenic and therapeutic significance. Ann NY Acad Sci 2008;1147:93–104.
[22] LeWitt PA. Levodopa therapeutics for Parkinson's disease: new developments. Parkinsonism Relat Disord 2009;15(Suppl. 1):S31–4.
[23] Rabey JM, Vered Y, Shabtai H, Graff E, Harsat A, Korczyn AD. Broad bean (*Vicia faba*) consumption and Parkinson's disease. Adv Neurol 1993;60:681–4.
[24] Farina E, Piu P, Strinna L. Extraction of L-DOPA from *Vicia faba* L. and other plants of the leguminous genera. Boll Soc Ital Biol Sper 1974;50:508–11.
[25] Raguthu L, Varanese S, Flancbaum L, Tayler E, Di Rocco A. Fava beans and Parkinson's disease: useful 'natural supplement' or useless risk? Eur J Neurol 2009;16:e171.
[26] Apaydin H, Ertan S, Ozekmekci S. Broad bean (*Vicia faba*) – a natural source of L-dopa – prolongs "on" periods in patients with Parkinson's disease who have "on–off" fluctuations. Mov Disord 2000;15:164–6.
[27] Heikkila RE, Hess A, Duvoisin RC. Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine in mice. Science 1984;224:1451–3.
[28] Wong SS, Li RH, Stadlin A. Oxidative stress induced by MPTP and MPP(+): selective vulnerability of cultured mouse astrocytes. Brain Res 1999;836:237–44.
[29] Greene LA, Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci USA 1976;73:2424–8.
[30] Kadota T, Yamaai T, Saito Y, Akita Y, Kawashima S, Moroi K, et al. Expression of dopamine transporter at the tips of growing neurites of PC12 cells. J Histochem Cytochem 1996;44:989–96.
[31] Mizuno Y, Sone N, Saitoh T. Effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 1-methyl-4-phenylpyridinium ion on activities of the enzymes in the electron transport system in mouse brain. J Neurochem 1987;48:1787–93.
[32] Schober A. Classic toxin-induced animal models of Parkinson's disease: 6-OHDA and MPTP. Cell Tissue Res 2004;318:215–24.
[33] Przedborski S, Tieu K, Perier C, Vila M. MPTP as a mitochondrial neurotoxic model of Parkinson's disease. J Bioenerg Biomembr 2004;36:375–9.
[34] Cassarino DS, Fall CP, Swerdlow RH, Smith TS, Halvorsen EM, Miller SW, et al. Elevated reactive oxygen species and antioxidant enzyme activities in animal and cellular models of Parkinson's disease. Biochim Biophys Acta 1997;1362:77–86.
[35] Lahaie-Collins V, Bournival J, Plouffe M, Carange J, Martinoli MG. Sesamin modulates tyrosine hydroxylase, superoxide dismutase, catalase, inducible NO synthase and interleukin-6 expression in dopaminergic cells under MPP-induced oxidative stress. Oxid Med Cell Longev 2008;1:54–62.
[36] Thomas B, Saravanan KS, Mohanakumar KP. In vitro and in vivo evidences that antioxidant action contributes to the neuroprotective effects of the neuronal nitric oxide synthase and monoamine oxidase-B inhibitor, 7-nitroindazole. Neurochem Int 2008;52:990–1001.
[37] Hartley A, Stone JM, Heron C, Cooper JM, Schapira AH. Complex I inhibitors induce dose-dependent apoptosis in PC12 cells: relevance to Parkinson's disease. J Neurochem 1994;63:1987–90.
[38] Shang T, Kotamraju S, Kalivendi SV, Hillard CJ, Kalyanaraman B. 1-Methyl-4-phenylpyridinium-induced apoptosis in cerebellar granule neurons is mediated by transferrin receptor iron-dependent depletion of tetrahydrobiopterin and neuronal nitric-oxide synthase-derived superoxide. J Biol Chem 2004;279:19099–112.
[39] Ryu EJ, Angelastro JM, Greene LA. Analysis of gene expression changes in a cellular model of Parkinson disease. Neurobiol Dis 2005;18:54–74.
[40] Sakakibara M, Mori K. Facile synthesis of (22R,23R)-homobrassinolide. Agric Biol Chem 1982;46:2769–79.
[41] McMorris TC, Chavez RG, Patil PA. Improved synthesis of brassinolide. J Chem Soc Perkin Trans 1 1996:295–302.
[42] Ramirez JA, Teme Centurion OM, Gros EG, Galagovsky LR. Synthesis and bioactivity evaluation of brassinosteroid analogs. Steroids 2000;65:329–37.
[43] Korzeniewski C, Callewaert DM. An enzyme-release assay for natural cytotoxicity. J Immunol Methods 1983;64:313–20.
[44] Martin A, Clynes M. Acid phosphatase: endpoint for in vitro toxicity tests. In Vitro Cell Dev Biol 1991;27A:183–4.
[45] Decker T, Lohmann-Matthes ML. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunol Methods 1988;115:61–9.
[46] Gelinas S, Martinoli MG. Neuroprotective effect of estradiol and phytoestrogens on MPP^+-induced cytotoxicity in neuronal PC12 cells. J Neurosci Res 2002;70:90–6.
[47] Takatsuto S, Ikekawa N. Remote substituent effect on the regioselectivity in the Baeyer–Villiger oxidation of 5-alpha-cholestane-6-one derivatives. Tetrahedron Lett 1983;24:917–20.
[48] Sharpless KB, Amberg W, Bennani YL, Crispino GA, Hartung J, Jeong K-S, et al. The osmium-catalyzed asymmetric dihydroxylation: a new ligand class and a process improvement. J Org Chem 1992;57:2768–71.
[49] Mitsunobu O, Yamada M, Mukaiyama T. Preparation of esters of phosphoric acid by the reaction of trivalent phosphorus compounds with diethyl azodicarboxylate in the presence of alcohols. Bull Chem Soc Jpn 1967;40:2380–2.
[50] Martin S, Dodge JA. Efficacious modification of the Mitsunobu reaction for inversions of sterically hidered secondary alcohols. Tetrahedron Lett 1991;32:3017–20.
[51] Bournival J, Quessy P, Martinoli MG. Protective effects of resveratrol and quercetin against MPP(+)-induced oxidative stress act by modulating markers of apoptotic death in dopaminergic neurons. Cell Mol Neurobiol 2009;29:1169–80.
[52] Ikekawa N, Takatsuto S, Kitsuwa T, Saito H, Morishita T, Abe H. Analysis of natural brassinosteroids by gas chromatography and gas chromatography-mass spectrometry. J Chromatogr 1984;290:289–302.
[53] Abe H, Morishita T, Uchiyama M, Takatsuto S, Ikekawa N, Ikeda M, et al. Occurrence of three new brassinosteroids: brassinone, (24S)-24-ethylbrassinone and 28-norbrassinolide, in higher plants. Experientia 1983;39:351–3.
[54] Schmidt J, Spengler B, Yokota T, Nakayama M, Takatsuto S, Voigt B, et al. Secasterone, the first naturally occurring 2,3-epoxybrassinosteroid from *Secale cereale*. Phytochemistry 1995;38:1095–7.
[55] Malikova J, Swaczynova J, Kolar Z, Strnad M. Anticancer and antiproliferative activity of natural brassinosteroids. Phytochemistry 2008;69:418–26.
[56] Misharin AY, Mehtiev AR, Zhabinskii VN, Khripach VA, Timofeev VP, Tkachev YV. Toxicity of (22R, 23R)-22,23-dihydroxystigmastane derivatives to cultured cancer cells. Steroids 2010;75:287–94.
[57] Jansen PJ, Lütjohann D, Abildayeva K, Vanmierlo T, Plösch T, Plat J, et al. Dietary plant sterols accumulate in the brain. Biochim Biophys Acta 2006;1761:445–53.
[58] Fricke CB, Schroder M, Poulsen M, von Bergmann K, Wester I, Knudsen I, et al. Increased plant sterol and stanol levels in brain of Watanabe rabbits fed rapeseed oil derived plant sterol or stanol esters. Br J Nutr 2007;98:890–9.
[59] Heverin M, Bogdanovic N, Lütjohann D, Bayer T, Pikuleva I, Bretillon L, et al. Changes in the levels of cerebral and extracerebral sterols in the brain of patients with Alzheimer's disease. J Lipid Res 2006;45:186–93.

[60] Schiepers OJ, de Groot RH, van Boxtel MP, Jolles J, de Jong A, Lütjohann D, et al. Consuming functional foods enriched with plant sterol or stanol esters for 85 weeks does not affect neurocognitive functioning or mood in statin-treated hypercholesterolemic individuals. J Nutr 2009;139:1368–73.
[61] Muthuraman P, Srikumar K. Induction of hexokinase I expression in normal and diabetic rats by a brassinosteroid isoform. Eur J Pharm Sci 2010;41:1–9.
[62] Ikekawa N, Zhao Y-J. Application of 24-epibrassinolide in agriculture. ACS Symp Ser 1991;474:280–91.
[63] Murkunde YV, Murthy PB. Developmental toxicity of homobrassinolide in Wistar rats. Int J Toxicol 2010;29:517–22.